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ORIGINAL RESEARCH ARTICLE

Effect of processing conditions on the bioactive compounds and biological properties of bee pollen

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Bee pollen has had a valued place in traditional medicine for centuries, even though its use in modern medicine is still limited by the lack of scientific evidence. In this study, we characterized and compared nine types of bee pollen, submitted to two preservation methods (lyophilization and drying), in relation to the content of phenolic compound and total flavonoids. The biological properties were also evaluated and compared. Pollen analysis allowed us to classify two samples as heterofloral, whilst the remaining were monofloral. The concentrations of flavonoid and phenolic compounds were greater in the lyophilized samples. Even though all samples presented antimicrobial activity, the lyophilized ones induced stronger inhibition against both Gram-negative and Gram-positive bacteria. For the yeasts studied, the difference between the two preservation procedures was not so relevant. The antioxidant activity was also greater in the lyophilized samples. Antimutagenic capacity was observed in all samples, independently of the preservation method, even though some bee pollen types decreased the number of gene conversion and mutant colonies more efficiently. In conclusion, the results suggest that lyophilization is better to preserve the bioactive compounds and biological properties of this natural product.

Efecto de las condiciones de procesado sobre los compuestos bioactivos y las propiedades biológicas del polen de abeja

El polen de abeja ha tenido un lugar valioso en la medicina tradicional durante siglos, aunque su uso en la medicina moderna sigue siendo limitado por la falta de evidencia científica. En este estudio se caracterizaron y compararon nueve tipos de polen de abeja, sometidos a dos métodos de conservación (liofilización y secado), en relación con el contenido de compuestos fenólicos y flavonoides totales. Las propiedades biológicas también fueron evaluadas y comparadas. El análisis del polen nos permitió clasificar dos muestras como heteroflorales, mientras que las restantes fueron monoflorales. Las concentraciones de flavonoides y compuestos fenólicos fueron mayores en las muestras liofilizadas. A pesar de que todas las muestras presentaron actividad antimicrobiana, las liofilizadas indujeron una inhibición más fuerte contra las bacterias Gram-negativas y Gram-positivas. Para las levaduras estudiadas, la diferencia entre los dos procedimientos de conservación no fue tan relevante. La actividad antioxidante fue también mayor en las muestras liofilizadas. Se observó capacidad antimutagénica en todas las muestras, independientemente del método de preservación, aunque algunos tipos de polen de abeja redujeron el número de conversión de genes y de colonias mutantes de manera más eficiente. En conclusión, los resultados sugieren que la liofilización es mejor para conservar los compuestos bioactivos y las propiedades biológicas de este producto natural.

Keywords: antimicrobial activity; antimutagenic activity; antioxidant activity; bee pollen; food preservation

Introduction

Bee pollen (BP) results from the agglutination of pollen from various selected flower species with small amounts of nectar or honey and salivary substances, which is carried out by the honey bee *Apis mellifera* (De-Melo & Almeida-Muradian, 2011). Bee pollen is a complete food product, being one of the few foods that possesses all the essential amino acids, but also containing carbohydrates (being glucose and fructose the most abundant), lipids, fibers, minerals, and oligominerals (Komosinska-Vassev, Olczyk, Kaźmierczak, Mencner, & Olczyk, 2015). Other constituents are vitamins, enzymes, coenzymes, and phenolic compounds, such as flavonoids, carotenoids, phytosterols, and terpenes (Campos, Olena, & Anjos, 2016; De-Melo et al., 2016).

The composition of this natural product depends on diverse factors including the botanical and geographical origin, harvest time, environmental, and climatic conditions, plant age (Estevinho, Rodrigues, Pereira, & Feás, 2012) and the beekeeping techniques and extraction methods (Feás, Vázquez-Tato, Estevinho, Seijas, & Iglesias, 2012). Chemical composition is also influenced by the preservation methods used, especially if these involve heat, as well as by the storage period (Almeida-Muradian, Pamplona, Coimbra, & Barth, 2005).

Bee pollen has been used in traditional medicine for a variety of purposes including treatment of prostatic conditions, wound healing, prevention of hay fever, relief of premenstrual syndrome, and climacteric symptoms associated with menopause (Münstedt, Voss, Kullmer, Schneider, & Hübner, 2015). Recently, bee products have drawn particular interest due to their presumed therapeutic properties, what is reflected in BP world production, nowadays reaching around 1500 tons per year (Estevinho et al., 2012). Among those properties, it is worth mentioning antibacterial, antifungal, anti-inflammatory, anticancer, anti-radiation, antiviral, antiallergic, and immunomodulatory (Fatrcová-Šramková et al., 2013; Feás et al., 2012).

Despite its promising applications, it is important to state that in nature, BP contains 20–30% water and a water activity between 0.66 and 0.82, being a favorable matrix for the proliferation of microorganisms and to the occurrence of chemical and enzymatic reactions (Campos et al., 2008). As such, the use of conservation techniques that allow an increase in BP shelf life is a generalized practice. Among those, hot air drying is the most frequently used in commercial BP, because of the reasonable process time, better sanitary conditions, and control of the drying conditions (Barajas-Ortiz, Martínez, & Rodríguez-Sandoval, 2011).

Another preservation technique, whose range of applications has mushroomed recently, is lyophilization. Even though the latter is considered the gentlest drying method (Ciurzyńska & Lenart, 2011), there is little evidence regarding its application on bee pollen. In fact, the existing studies are recent and limited to Brazilian bee pollen samples (De-Melo et al., 2016). In this context, in order to compare preservation methods concerning their efficiency but also the changes on the BP characteristics, we evaluated the effect of drying and lyophilization on the content of bioactive compounds, specifically total phenolic compounds, and flavonoids, as well as in the biological activities, particularly antioxidant, antimicrobial, and anti-mutagenic, of nine types of bee pollen.

Materials and methods

Bee pollen sampling and preparation

In this study, nine organic BP samples harvested in Portugal in 2014, submitted to two preservation processes: lyophilization ($n = 9$) and drying ($n = 9$), were analyzed. Organic honey is produced in an ecologically based system that promotes the use of good agricultural practices in order to maintain the agricultural ecosystem balance and diversity, encouraging the sustainable use of natural resources, environmental quality, animal welfare and, to a later extent, human health (EU Council Directive 834/EU Council Directive, 2007). Identification of the bee pollen types was carried out by optical microscopy using the reference collection of the Escola Superior Agrária de Bragança – Instituto Politécnico de

Bragança and different pollen morphology guides and international databases.

In order to ensure the good quality and safety of the samples, these were studied regarding the presence of aerobic mesophiles, molds, and yeasts, fecal coliforms, *Escherichia coli*, clostridia spores, *Bacillus cereus*, and *Salmonella* spp. and *Staphylococcus aureus*, as described by Feás et al. (2012). The values obtained for all samples were below the stated on the hygienic standards (Campos et al., 2008).

Each sample was divided into two aliquots. One was frozen at −40 °C and dehydrated for 18 h in a vacuum lyophilizer (Labconco, Freezone 4.5, USA); while the other was thawed at room temperature and then dried in an electric oven with forced air circulation (ESA 1368, Sercon) at 42 °C, between 24 and 52 h. This variation on the processing time occurred because trays were removed when the moisture of the samples reached 6–8%. Afterwards, the samples were vacuum packed in food-grade polyethylene bags, stored at room temperature and protected from light until further analysis, which were performed not later than 30 days. The methanolic BP extracts were obtained according to the method previously described in detail by Morais, Moreira, Feás, and Estevinho (2011).

Reagents

Trolox, Fluorescein, 2,2′-Azobis (2-methylpropionamidine) dihydrochloride (AAPH), gallic acid, quercetin, 3,5-Di-tert-4-butylhydroxytoluene (BHT), linoleic acid (LA), α-tocopherol, β-carotene, Folin–Ciocalteu reagent, 2,3,5-triphenyl tetrazolium chloride (TTC), were obtained from Sigma Chemical Co (St. Louis, MO, USA). Methanol, chloroform ($CHCl_3$), sodium carbonate (Na_2CO_3), sorbitan monooleate (Tween 40), gentaminice, and fluconazol were obtained from Merck (Darmstadt, Germany). High purity water (18 MX cm) was obtained from a Milli-Q purification system (Millipore, Barnstrad, MA, USA).

Total phenolic and flavonoid compounds

Total phenolics

Firstly, the dried methanolic extract of each BP sample was diluted in methanol (1:10 g/ml). Then, 500 μl of the diluted solutions were mixed with 500 μl of Folin–Ciocalteu reagent and 500 μl of Na_2CO_3 (10% w/v). Mixtures were stored in the dark at room temperature for 1 h and then, absorbance was measured at 700 nm in a spectrophotometer (Varian UV–vis, Cary 50 Scan). The blank (negative control) was prepared under the same conditions but without the diluted solution of the sample extract. A gallic acid standard curve (concentrations from 0.01 to 0.7 mM) was constructed and results were expressed as mg of gallic acid equivalents (GAE) per g of bee-pollen dried extract.

Flavonoid compounds

Flavonoid content was determined as described by Feás et al. (2012). An aliquot (250 µl) of the diluted solution of dried sample extract was mixed with 1.25 ml of distilled water and 75 µl of NaNO$_2$ solution at 5%. After 5 min of rest, 150 µl of AlCl$_3$·H$_2$O at 10% were added and, 6 min later, 500 µl of NaOH (1 M) and 275 µl of distilled water. The intensity of the mixture's color was quantified at 510 nm. Quercetin was used in the determination of the standard curve and results were expressed as mg of quercetin equivalents (QE) per g of pollen dried extract.

Antioxidant activity

In order to avoid possible interferences and considering the complex nature of bee pollen constituents the antioxidant activity was determined using two methodologies, as recommended by Sakanaka and Ishihara (2008). The two analytical methods applied were β-carotene bleaching (BCB) assay and oxygen radical absorbance capacity (ORAC) assay.

BCB assay

This experiment was carried out according to the method described by Ahn, Kumazawa, Hamasaka, Bang, and Nakayama (2004) with some modifications. Firstly, 5 mg of β-carotene were dissolved in 25 ml of CHCl$_3$. Four milliliters of this solution was pipetted into a 100 ml round-bottom flask and CHCl$_3$ was removed under vacuum. Then, 80 mg of linoleic acid, 800 mg of Tween 80 emulsifier and 200 ml of distilled water were added; 4.8 ml of this mixture were transferred into test tubes containing 200 µl of different concentrations of the pollen extracts. The tubes were shaken and incubated in a water bath at 50 °C. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm. Absorbance readings were then recorded at 20-min intervals until the control sample had changed color. A blank, without β-carotene, was prepared for background subtraction. Lipid peroxidation (LPO) inhibition was calculated using the equation:

$$\text{LPO inhibition} = (\text{-carotene content after 2 h} / \text{initial -carotene content}) \times 100.$$

The extract concentration providing 50% of antioxidant activity (EC$_{50}$) was calculated by interpolation from the graph of antioxidant activity percentage against extract concentration. As standard it was used BHT, at a concentration of 40 mg/ml.

ORAC activity assay

This assay was carried out according to Arruda, Pereira, Estevinho, and Almeida-Muradian (2013), using an automated plate reader (Thermo Scientific™ Multiskan™ Microplate Photometer, EUA) with 96-well plates.

Several concentrations (1.5–25 mg/ml) of the each BP sample extract solution were prepared with phosphate buffer (75 mM, pH 7.4). Peroxyl radical was generated using 2,2′-azobis (2-amino-propane) dihydrochoride (AAPH) aqueous solution (153 mM). Fluorescein was used as substrate (40 nM). ORAC analyzer was programmed to record the fluorescence every minute after addition of AAPH and all measurements were expressed in relation to the initial reading. Fluorescence analysis conditions were excitation at 493 nm and absorption at 515 nm. The standard curve was linear between 6.25 and 100 µM of Trolox. Results were calculated from the different areas under the fluorescence decay curves between the blank and the sample and expressed as µM of Trolox equivalents (TE)/g of bee pollen extract.

Antimicrobial activity

The antimicrobial effect of bee pollen extracts was evaluated against microorganisms isolated at the Local Health Units of Bragança and Mirandela and identified at the Laboratory of Microbiology of the School of Agriculture of Bragança. The tests involved four bacteria: two Gram-negative bacteria (Extended-spectrum β-lactamases producing *Klebsiella pneumoniae* ESA36 and multidrug-resistant *Pseudomonas aeruginosa* ESA38); two Gram-positive (Vancomycin-resistant *Enterococcus* ESA5 and Methicillin-resistant *Staphylococcus aureus* ESA77). The yeasts used were *Candida parapsilosis* ESA70 and *Candida glabrata* ESA11. These microorganisms were selected since, according to the recent prevalence survey of the Centers for Disease Control and Prevention, these are among the most common causative pathogens of healthcare associated infections.

The minimum inhibitory concentration (MIC) was determined according to the described by Morais et al. (2011) using Brain Heart Infusion or Yeasts Peptone Dextrose on microplate (96 wells). The inoculum was prepared by diluting cell mass in 0.85% sodium chloride solution and adjusted to 0.5 Mac Farland scale, confirmed spectrophotometrically at 540 nm for bacteria and 640 nm for yeasts. Sample extract solution was diluted in DMSO and transferred into the first draw-well and serial dilutions were performed. The inoculums, 10^8 for bacteria and 10^5 for yeasts, were added to all wells and the plates were incubated at 37 °C for 24 h (gram-negative and gram-positive bacteria) or 25 °C for 48 h (yeasts). A DMSO control (DMSO with inoculated medium) was also introduced. Antimicrobial activity was detected by adding 20 µl of 1% 2,3,5-triphenyl-2H-tetrazolium chloride (TTC) solution. The MIC was defined as the lowest concentration of dried extract that inhibited visible growth, as indicated by TTC staining. The assays were performed in triplicate for each microorganism.

Antimutagenic activity

The determination of the antimutagenic activity of the 9 types of bee pollen that underwent two conservation processes (drying or lyophilization) was performed using yeast cells (D7 diploid strain of *Saccharomyces cerevisiae* ATCC 201137) according to the recommended by Zimmermann (1984). It were evaluated three pollen extract concentrations (0, 0.5, and 1.0%) in 0.5% ethyl methanesulfonate (EMS), a mutagenic compound.

Prior to each experiment, *S. cerevisiae* D7 strain (MATa/MATa, ade2-40/ade 2-119, trp 5-12/ trp 5-27, ilv 1-92/ilv 1-92) was tested for the frequency of spontaneous convertants at the tryptophan (trp) locus and revertants at the isoleucine (ilv) locus. Cells from culture with low spontaneous gene conversion and reverse point mutation frequencies were grown in a liquid medium at 28 °C until the stationary growth phase. Yeast cells were pelleted and re-suspended in a volume of 0.1 M sterile potassium phosphate buffer, pH 7.4, to obtain the final cell concentration of 2×10^8/ml. The mixture (4 ml) contained 1 ml of cell suspension, potassium phosphate buffer and methanolic extracts of bee pollen, reaching the final concentrations of 0.00, 0.25, 0.50, and 0.75 mg/l. The mixture was incubated for 2 h at 37 °C and cells were plated to ascertain survival, trp convertants and ilv revertants. EMS was used as control; all tests were performed in triplicate.

Statistical analysis

All experiments were performed in triplicate, assuring sample groups of equal size. Possible correlations among analytical variables were assessed using *R*-Pearson correlation coefficient. Prior the use of ANOVA methodology, it was verified if the groups had variances homogeneity, using the Levene test. To verify if the ANOVA model had adjusted data correctly, the model residuals were fitted in a qq-plot in order to verify if they assume normal distributed, which confirmed the ANOVA good model fit to the original data. The *p*-values less than or equal to 0.05 were considered statistically significant.

Results

BP analysis

The results of the pollen analysis showed that two samples (Poly1 and Poly2) are heterofloral, presenting pollen granules with a wide variety of colors that correspond to different types of pollen and, therefore, plant species. The remaining seven samples exhibited a percentage above 90% of a certain type of pollen and were classified as monofloral: *Rubus* spp. (Rosaceae), *Castanea sativa* (Fagacea), *Cistus* spp. (Cistaceae), *Cytisus* spp. (Fabaceae), *Eucaliptus* spp. (Myrtaceae), *Echium* spp. (Boraginaceae), and *Erica* spp. (Ericaceae). The identified families are very common in Portugal. Indeed, these results are in agreement with the observed by Morais et al. (2011), who reported that the dominant botanical families in Trás-os-Montes are Rosaceae, Leguminosae, Fagaceae, Boraginaceae, Labiatae, and Ericaceae.

Total phenolic and flavonoid compounds

The total phenolic and flavonoid compounds determined on the BP samples are presented in Table 1. From the two-way analysis of variance (ANOVA), it was verified that the interaction term between the processes of preservation and type of pollen was significant (*p*-value < 0.05), suggesting that the variation found in the total phenolics and flavonoids was dependent on the two factors. Previously, in order to validate the use of that methodology, it was confirmed that data had homogeneity of variances and that residuals had normal distribution in qq-plot.

The general comparison shows that the concentration of phenolic compounds determined on the samples *Erica* spp. and *Castanea* spp. were significantly higher than the quantified on the other types of bee pollen (*p* < 0.05). On the lyophilized samples the concentrations of total phenols ranged from 16.67 ± 0.38 (sample Poly2) to 47.20 ± 1.99 mg GAE/g pollen (BP from *Erica* spp.). Regarding the dried BP, values were between 12.75 ± 0.25 and 35.05 ± 0.05 mg GAE/g pollen, on the same samples. Indeed, for all types of BP under study, the values of total phenolics of the dried sub-sample were significantly lower than the determined in the lyophilized one, suggesting that this conservation technique is more adequate to ensure the preservation of those bioactive compounds. These statistical differences may be assigned to the different procedures that the two preservation techniques involve: prior to lyophilization samples are frozen, whereas before the treatment on the electric oven dried samples are thawed. Those processes cause cellular damages with the release of distinct enzymes that oxidize phenolic compounds (Silva, Videira, Monteiro, Valentão, & Andrade, 2009).

The concentrations of phenolic compounds found on this study are in agreement with the reported by previous studies using dried bee pollen (Carpes et al., 2013; Morais et al., 2011). The studies available on lyophilized bee pollen are recent and almost only directed to Brazilian samples and also report compatible values (De-Melo et al., 2016). Concerning total flavonoids, significantly higher values were obtained for *Erica* spp. BP (10.25 ± 1.15 in lyophilized and 6.99 ± 0.33 mg QE/g pollen in dried subsample) and BP from *Castanea* spp. (8.13 ± 0.63 and 6.13 ± 0.46 mg QE/g pollen in lyophilized and dried, respectively). On the other hand, the sample classified as monofloral *Cytisus* spp. had significantly lower concentration of these compounds, for both processing techniques.

Unlike total phenolics, where the preservation process significantly influenced the concentration on all the samples, the flavonoid content did not differ depending

Table 1. Total bioactive compounds and antioxidant activity of lyophilized and dried bee pollen.

		Rubus spp.	Castanea sativa	Eucalyptus spp.	Cistus spp.	Cytisus spp.	Echium spp.	Erica spp.	Poly1	Poly2
Total phenols mg GAE/g pollen	Lyophilized	21.16 ± 2.22	43.85 ± 3.97	30.28 ± 2.06	27.62 ± 2.10	24.36 ± 3.19	25.85 ± 0.60	47.20 ± 1.99	18.87 ± 0.79	16.67 ± 0.38
	Dried	15.28 ± 2.46	29.90 ± 3.24	23.46 ± 2.57	21.00 ± 1.15	16.93 ± 0.88	19.30 ± 1.46	35.05 ± 0.05	14.33 ± 0.89	12.75 ± 0.25
	p-value	0.035	0.009	0.023	0.009	0.017	0.002	0.001	0.003	0.001
Total flavonoids mg QE/g pollen	Lyophilized	4.85 ± 0.53	8.13 ± 0.63	5.25 ± 0.50	5.06 ± 0.36	4.85 ± 0.53	6.62 ± 0.42	10.25 ± 1.15	5.48 ± 0.23	6.43 ± 0.50
	Dried	3.93 ± 0.83	6.99 ± 0.33	3.05 ± 0.65	2.96 ± 0.32	3.65 ± 0.66	4.15 ± 0.13	6.13 ± 0.46	3.57 ± 0.66	4.43 ± 0.14
	p-value	0.182	0.048	0.010	0.002	0.071	0.001	0.004	0.009	0.003
BCB assay EC$_{50}$ mg/g pollen	Lyophilized	3.18 ± 0.67	1.08 ± 0.01	1.08 ± 0.01	3.54 ± 0.09	1.17 ± 0.09	2.16 ± 0.03	0.71 ± 0.04	1.05 ± 0.25	2.23 ± 0.21
	Dried	5.01 ± 0.15	2.19 ± 0.01	2.16 ± 0.03	4.05 ± 0.29	2.63 ± 0.07	4.27 ± 0.11	3.19 ± 0.18	2.63 ± 0.07	4.27 ± 0.11
	p-value	0.010	0.001	0.001	0.044	0.000	0.001	0.001	0.001	0.001
ORAC assay mol eq. Trolox/g pollen	Lyophilized	189.99 ± 16.81	189.66 ± 4.93	266.26 ± 7.98	186.65 ± 11.14	221.94 ± 8.20	229.81 ± 8.09	149.94 ± 6.75	253.98 ± 9.44	284.69 ± 13.69
	Dried	292.51 ± 17.63	246.04 ± 8.54	351.30 ± 8.75	262.82 ± 9.23	323.62 ± 5.49	401.58 ± 6.41	241.40 ± 11.41	363.13 ± 9.53	403.30 ± 14.36
	p-value	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001

on the conservation technique for BPs from *Rubus* spp. ($p = 0.182$) and *Cytisus* spp. ($p = 0.071$). The concentration of flavonoids obtained for the dried samples is in agreement with the concentrations reported by Feás et al. (2012) and LeBlanc, Davis, Boue, DeLucca, and Deeby (2009). However, the values obtained by Carpes, Begnini, Alencar, and Masson (2007) in bee pollen from Brazil were higher (ranging from 2.10 to 28.33 mg GAE/ g pollen). These slight variations may be due, among other factors, to the different botanical and geographical origin of the studied samples (Carpes et al., 2007). As far as we know, there are no previous reports regarding the content of flavonoids in lyophilized bee pollen.

Antioxidant activity

According to the literature the accurate determination of the antioxidant activity is influenced by many factors and, in the particular case of bee pollen it cannot be evaluated by a single method due to the complexity of its constituents (Aličić, Šubarić, Jašić, Pašalić, & Ačkar, 2014).

BCB assay

In the BCB assay, the free radicals attack the highly unsaturated β-carotene, which, in the absence of an antioxidant, undergoes rapid discoloration. On the other hand, the presence of antioxidants minimizes the oxidation of the β-carotene conjugate system by hydroperoxides, which are neutralized by the antioxidants of the extract (Morais et al., 2011).

The mean values and associated errors obtained for all pollen samples using the BCB assay are shown in Table 1. Overall, the lyophilized samples had EC$_{50}$ values varying between 0.71 ± 0.04 (*Erica* spp.) and 3.54 ± 0.09 mg/g of BP (*Cistus* spp.), while the values for the dried samples were between 2.16 ± 0.03 (*Erica* spp.) and 5.01 ± 0.15 mg/g of BP (*Rubus* spp.). Indeed, generally, the EC$_{50}$ values obtained for lyophilized samples were significantly smaller than the achieved on the dried samples for all types of pollen under study ($p < 0.05$), what is consistent with the fact that the concentration of bioactive compounds is superior in the lyophilized samples when compared to the dried ones. The EC$_{50}$ results were dependent on the pollen type and processing conditions (interaction term in the two-way ANOVA was significant, presenting a p-value < 0.05).

The mismatch between the antioxidant activity estimated through the BCB assay and the content of bioactive compounds is in favor of the assumption that the composition of flavonoids and phenolic compounds of each sample is more important than their total content. Also, the lack of correlation between these variables corroborates that. Indeed, the high ability of polyphenols to neutralize active oxygen species is strongly as related to their specific structure, like the conjugated double bonds and the number of hydroxyl groups in the

aromatic ring (Aličić et al., 2014). This is in agreement with the reported by Morais et al. (2011), who did not find any correlation between antioxidant activity and the composition in polyphenols.

ORAC assay

In order to evaluate the differences between the two conservation processes and pollen types, a two-way ANOVA after ensuring the homogeneity of variances between groups (Levene test, p-value = 0.689) was used. Again, the influence of these two factors should be evaluated together because the interaction term between the two factors considered in the model was significant (p-value = 0.0019). The model showed a good fit to the evaluated data, confirmed by the normal distribution found in residual values. We found significant differences between the results obtained for the lyophilized and dried samples, with p-values > 0.002 for all the cases. The *Echium* pollen sample showed the greatest difference between lyophilized and dried sample, followed by the Poly2 sample. The lyophilized *Erica* spp. pollen sample presented the lowest values in the ORAC assays (149.94 ± 6.75 mol eq. Trolox/g pollen), followed by lyophilized *Castanea* pollen (186.65 ± 11.14 mol eq. Trolox/g pollen). The results of those two samples differ slightly from the others, whose values are statistically equal. For the dried samples it were obtained results with greater variability, suggesting greater differences in the phenolic compounds profile, perhaps due to possible losses of volatile compounds. Lower and statistically equal values of Trolox equivalents were obtained in the pollen of *Castanea* spp., *Cistus* spp., and *Erica* spp.

According to the literature, the antioxidant activity strongly depends on the profile of phenolic compounds (Campos et al., 2008; Feás et al., 2012). In this particular study, it was observed that the samples of *Erica* spp. and *Castanea* spp., which has the highest levels of total flavonoids and phenolic compounds, possess high activity ORAC values (it had also the highest antioxidant activity when BCB assay was applied). However, this match was not found for all types of samples, suggesting that the specific phenolic composition is more relevant than the total content since no correlation was found between ORAC (and also BCB assay) results and the levels of total flavonoids and phenolic compounds. Also, the possible contribution of other non-phenolic compounds on the antioxidant activity cannot be dismissed, which is in agreement with the stated by Markiewicz-Żukowska et al. (2013).

The results of the antioxidant activity obtained with the two methodologies are not always equivalent; for instance: the results obtained for *Castanea* spp. and *Eucalyptus* spp. were very similar when the BCB methodology was applied but are statistically different following the ORAC method. This may be justified by the different reaction mechanisms that are involved, and, consequently, the sample may exhibit high antioxidant activity when using a method and not the same result when using other.

Antimicrobial activities of BP samples

The increasing emergence of antibiotic-resistant pathogens turned the search for alternative tools against infectious diseases into a priority. In this study, the antimicrobial activity of the different types of bee pollen was tested against six agents of hospital-acquired infections. The MICs obtained are presented in Table 2. All microorganisms were inhibited by bee pollen, even though bacteria were more sensitive than yeasts, what is consistent with the described by previous studies (Bogdanov, 2012; Pascoal, Rodrigues, Teixeira, Feás, & Estevinho, 2014). The antibacterial activity varied significantly with the sample conservation procedure, for all the strains under study. The most effective in inhibiting bacterial growth was the lyophilized *Erica* spp. bee pollen, for which it were obtained the lowest MIC values: methicillin-resistant *Staphylococcus aureus* (1.58 ± 0.14 mg/ml), Vancomycin-resistant *Enterococcus* ESA5 (2.25 ± 0.25 mg/ml), *Pseudomonas aeruginosa* ESA38 (2.77 ± 0.46 mg/ml), *Klebsiella pneumoniae* ESA36 (3.92 ± 0.38 mg/ml). For dried pollen samples, it was also *Erica* pollen that caused higher inhibition, with MIC values following the order *S. aureus* < *Enterococcus* < *P. aeruginosa* < *K. pneumonia*. For both preservation processes and all kinds of pollen, Gram-negative bacteria showed greater resistance, probably due to the greater complexity of their cell wall.

Concerning yeasts, the highest antifungal activity was recorded in the lyophilized sample of *Castanea sativa*: *C. parapsilosis* (10.15 ± 1.31 mg/ml) and *C. glabrata* (12.17 ± 1.53 mg/ml). In general, the highest MIC values were obtained for BP Poly 2. The profile of inhibition of *C. parapsilosis* and *C. glabrata* was similar on the different pollen types and was not statistically different. Also, the antifungal effect of BP was less influenced by the processing conditions when compared to the above described for bacteria.

These results combined with others already available on literature suggest that bee pollen extracts may be useful as a complement to the conventional antimicrobial agents against resistant pathogens, since, as far as we know, it has not yet been discovered resistance to the action of bee pollen (Pascoal et al., 2014).

Antimutagenic activity

According to the literature, the study of the antimutagenic potentials of traditionally natural products constitutes an important approach for the discovery of novel chemotherapeutic drugs, being this beneficial activity mostly related to the presence of various secondary metabolites such as flavonoids (Ghazali et al., 2011). In this work, the antigenotoxic effect of bee pollen on *Saccharomyces cerevisiae* was tested with 3 levels of pollen extract concentrations

Table 2. Antimicrobial activity, expressed as MIC (mg/mL), of the different methanolic extracts of lyophilized and dried bee pollen.

		Enterococcus[a]	*Staphylococcus aureus*[b]	*Klebsiella pneumoniae*[c]	*Pseudomonas aeruginosa*[d]	*Candida parapsilosis*	*Candida glabrata*
Rubus spp.	Lyophilized	4.70 ± 0.92	3.58 ± 0.38	6.58 ± 1.13	5.75 ± 0.90	12.37 ± 1.71	15.50 ± 2.00
	Dried	7.25 ± 0.66	6.00 ± 0.50	9.25 ± 1.15	8.25 ± 0.90	14.33 ± 1.61	16.67 ± 1.44
	p-value	0.017	0.003	0.044	0.027	0.220	0.459
Castanea sativa	Lyophilized	3.04 ± 0.45	2.03 ± 0.46	4.37 ± 0.55	3.50 ± 0.43	10.15 ± 1.31	12.17 ± 1.53
	Dried	4.96 ± 0.70	3.92 ± 0.38	7.11 ± 0.35	5.57 ± 0.40	12.50 ± 2.50	16.33 ± 0.76
	p-value	0.016	0.005	0.002	0.004	0.223	0.013
Cistus spp.	Lyophilized	3.57 ± 0.81	2.60 ± 0.75	4.50 ± 0.25	4.17 ± 0.52	10.47 ± 0.55	13.33 ± 1.53
	Dried	5.17 ± 0.76	5.37 ± 0.70	6.08 ± 0.95	5.72 ± 0.70	15.58 ± 1.38	15.57 ± 1.78
	p-value	0.068	0.010	0.039	0.037	0.004	0.174
Eucalyptus spp.	Lyophilized	3.37 ± 0.42	2.75 ± 0.90	4.42 ± 0.38	3.83 ± 0.14	11.08 ± 1.01	13.25 ± 0.90
	Dried	5.67 ± 0.76	4.83 ± 0.58	6.63 ± 0.32	7.33 ± 1.04	17.23 ± 1.55	19.25 ± 0.66
	p-value	0.010	0.028	0.002	0.004	0.005	0.011
Cytisus spp.	Lyophilized	3.68 ± 0.54	2.27 ± 0.26	5.42 ± 0.52	4.25 ± 0.25	10.97 ± 0.50	16.50 ± 1.00
	Dried	5.38 ± 0.13	4.58 ± 0.52	7.48 ± 0.28	5.83 ± 0.52	14.07 ± 1.10	17.42 ± 0.63
	p-value	0.006	0.002	0.032	0.009	0.011	0.250
Echium spp.	Lyophilized	3.75 ± 0.25	2.53 ± 0.68	6.20 ± 0.26	4.75 ± 0.25	11.00 ± 1.50	14.07 ± 1.10
	Dried	2.58 ± 1.09	5.73 ± 0.64	8.42 ± 0.80	7.08 ± 1.01	13.57 ± 2.57	18.15 ± 3.25
	p-value	0.323	0.004	0.011	0.018	0.210	0.152
Erica spp.	Lyophilized	2.25 ± 0.25	1.58 ± 0.14	3.92 ± 0.38	2.77 ± 0.46	11.83 ± 0.76	12.33 ± 0.76
	Dried	3.16 ± 0.52	2.84 ± 0.76	5.33 ± 0.65	4.25 ± 0.50	11.63 ± 0.81	16.33 ± 0.76
	p-value	0.052	0.048	0.031	0.002	0.771	0.091
Poly1	Lyophilized	5.08 ± 0.52	4.08 ± 0.29	6.92 ± 0.14	5.83 ± 0.38	11.23 ± 1.66	15.50 ± 1.50
	Dried	7.42 ± 0.80	6.28 ± 0.62	9.67 ± 1.26	8.85 ± 1.13	14.33 ± 1.04	17.00 ± 0.50
	p-value	0.013	0.005	0.024	0.012	0.052	0.176
Poly2	Lyophilized	5.35 ± 0.33	4.52 ± 0.25	7.67 ± 1.04	6.25 ± 0.90	12.37 ± 1.71	18.25 ± 1.30
	Dried	7.50 ± 1.00	5.45 ± 0.51	9.83 ± 0.29	7.48 ± 1.00	15.17 ± 2.25	19.81 ± 0.60
	p-value	0.024	0.047	0.025	0.188	0.161	0.132

[a]Vancomycin-resistant *Enterococcus*.
[b]Methicillin-resistant *Staphylococcus aureus*.
[c]Extended-spectrum β-lactamases producing *Klebsiella pneumoniae*.
[d]Multidrug-resistant *Pseudomonas aeruginosa*.

(0, 0.5, and 1.0%) in a 0.5% EMS concentration. This compound produces random mutations in genetic material by nucleotide substitution, being an alkylating agent. The data obtained in these experiments regarding the percentage of survivals, gene conversion colonies and reverted mutations colonies are shown in Figure 1.

It is possible to infer that EMS efficiently induced mutations and decreased yeast colony survival considering the statistical difference obtained between the control (where EMS was not included) and the other assays. The survival and rate of mutations was practically constant among the assays, suggesting that there was good repeatability. The profile of survivals obtained using the different types of lyophilized and dried samples was similar: lower survival values corresponding to lyophilized BP and to higher extract concentrations, what may be related to the reported antifungal effect of this product.

The percentages of colony survival (%) were lower, yet not statistically different, using *Erica* sp. bee pollen (33.71 and 38.93% of survivals for lyophilized and dried samples, respectively), followed by BP from *Castanea* sp., *Eucalyptus* sp. This suggests that the extracts of those types of bee pollen may have some characteristics that favor the reduction of *S. cerevisiae* colonies, in both conservation processes, even for lower concentrations.

The amount of colonies with converted genes in the control group (without EMS) was, as expected, very low ($0.77 ± 0.3 × 10^5$ UFC/ml). When EMS, an alkylating agent, was included on the assays, without including BP extract, the number of gene conversion colonies increased notably, reaching $52.0 × 10^5 ± 2.0$ UFC/ml, with good repeatability among assays.

The addition of BP extract, at concentrations of 0.5 and 1.0%, allowed studying the anti-mutagenic effect of this natural product. The seven pollen samples that presented greater anti-mutagenic effect, were in ascending order: *Castanea* (dried, C = 1%; GCC = $21.5 × 10^5$ UFC/ml); *Erica* (lyophilized, C = 0.5%; GCC = $21.4 × 10^5$ UFC/ml); *Cistus* (C = 1%; GCC = $19.5 × 10^5$ UFC/ml); *Erica* (dried, C = 1%; GCC = $19.1 × 10^5$ UFC/ml); *Eucaliptus* (lyophilized, C = 1%, GCC = $17.7 × 10^5$ UFC/ml); *Castanea* (lyophilized sample, C = 1%; GCC = $16.4 × 10^5$ UFC/ml); *Erica* (lyophilized sample, C = 1%; GCC = 11.7 UFC/ml). As expected, superior concentrations of *Erica* pollen extract, followed by *Castanea* and *Eucalyptus* pollen extracts, possessed highest protective effect translated by lowest number of gene conversion colonies.

The lyophilized bee pollen, for all botanical types, was significantly more able to decrease the amount of gene conversion colonies, what suggests, indirectly, that
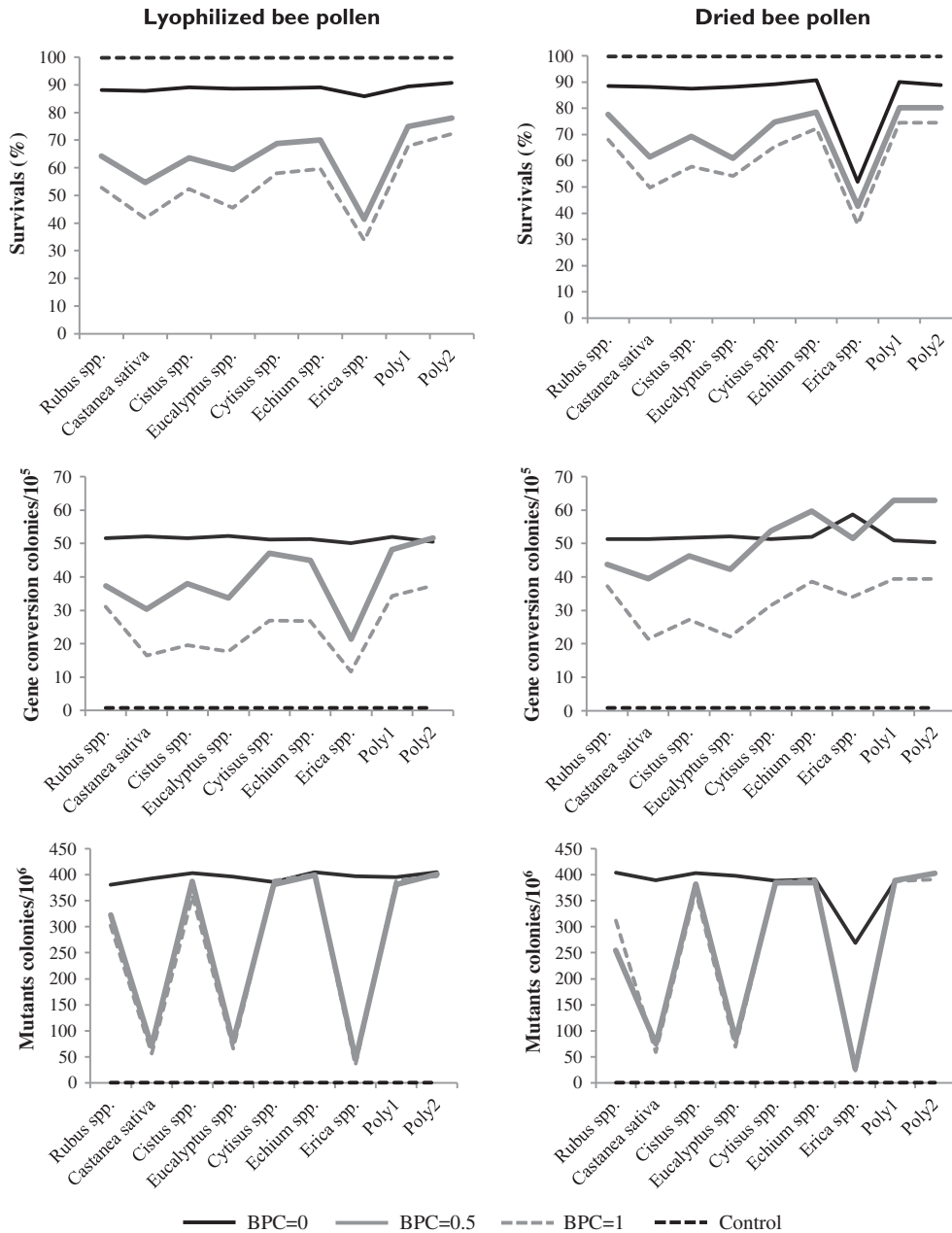
Figure 1. Antimutagenic activity parameters of the 9 bee pollen extracts at different concentrations, for the two processing conditions (lyophilized and dried).

lyophilization is better for preserving the anti-mutagenic effect of BP when compared to drying. The reversion of mutant colonies observed on the control assay was very low (0.44 ± 0.03 × 10^6 CFU/ml), what was already expected, since no mutagenic agent was applied (0% EMS). The tests performed without the BP extract (BPC = 0) gave the highest values of mutant colonies, with a mean value and associated error of 396 ± 9 × 10^6 CFU/ml, statistically equal among assays. The figure shows that BP samples of *Castanea*, *Erica*, and *Eucaliptus* were those with highest capacity of reverting mutations, what is consistent with the results obtained for the above described biological activities. However, it

is worth referring that there were no differences between the antimutagenic activities of the samples submitted to the two conservation processes (*p*-value > 0.05).

Discussion

The content of total phenolic, flavonoid compounds, and antioxidant activity was higher in the lyophilized pollen, for all the types under study. All pollen samples presented antimicrobial activity, especially against bacteria, suggesting that this product is promising as a complement in the treatment of drug-resistant microorganisms.

However, it is noteworthy that the lyophilized bee pollen induced a stronger inhibition when compared to the dehydrated samples against Gram-negative and Gram-positive bacteria. All samples had antimutagenic activity that differed amongst the bee pollen botanical types. However, this biological property did not vary depending on the preservation conditions.

Our results suggest that lyophilization may allow better preservation of the chemical characteristics and biological functions of bee pollen, when compared to drying. However, further studies using more samples and pollens with different botanical and geographical origins are necessary to confirm the present data.

Disclosure statement

No potential conflict of interest was reported by the authors.

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